MAMMALIAN LIMB MORPHOLOGY: THE ROLE OF THE CRAWL IN DETERMINING MARSUPIAL LIMB INTEGRATION, DEVELOPMENT, AND EVOLUTION

BY

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THESIS

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ABSTRACT

Marsupials and eutherians, though closely related sister taxa, differ greatly in the variety of morphologies found within their groups. This inequality has largely been argued to be due to the reproductive differences. The crawl most marsupial neonates complete after birth has constrained the evolution of the forelimb and the group as a whole. However, the hind limb, which does not participate in the crawl, may have "escaped" the crawl's constraint. This strong selective pressure to develop the forelimb early has also led to a decrease in integration (i.e. covariance) between the serially homologous elements of the limb. While limb integration has been well studied in adults, there exists study quantifying developmental integration in any system, which is unfortunate as it is important to examine integration across contexts (genetic, developmental, functional) in order to understand the evolution of morphologies. The purpose of this study is to determine both if marsupials are more likely to adapt their hind limbs than their forelimbs as well as to determine developmental patterns of limb integration to better understand the role of the crawl in marsupial evolution. To determine whether or not marsupials are more likely to adapt their hind limbs, length and width measurements were taken from the skeletal elements of the girdles, stylopod, zeugopod, and autopod from 196 therian taxa (82 marsupials, 114 eutherians). This data were then incorporated into principal component analyses and correlation matrices compared with the species' functional group. Marsupials were found to be less likely to specialize their forelimbs than eutherians and while also more likely to specialize their hind limbs than forelimbs, still less likely to specialize their hind limbs than eutherians. In order to determine developmental integration, embryos at different but comparable stages were cleared and stained to visualize bone and cartilage growth from three marsupials (*Monodelphis domestica, Sminthopsis macroura, Trichosurus vulpecula*) and one eutherian (*M. musculus*). A reflex scope was used to place four points on the stylopod, zeugopod, and autopod and these points were used to determine gross length and width for each element. A combination of correlation and covariance matrices, Mantel's test, and Mann-Whitney U tests were used to determine the integration patterns, both within and between the limbs, for each embryonic stage. Integration patterns significantly differed between stages and for marsupials, between limb integration decreased over time. Overall this study found that the hind limb of marsupials has been constrained by the crawl and that developmental integration changes between stages, suggesting a post-birth period of limb flexibility in both marsupials and eutherians.

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INTRODUCTION

The vertebrate limb structure is a well-studied model system for understanding the interplay between genetics, development, function, and selection in the creation of new morphologies. Marsupials provide an excellent natural system to study how new limb morphologies evolve as compared to the more commonly studied mammals, the eutherians. Marsupials are closely related to eutherians mammals and contain species that are highly morphologically convergent with eutherians (e.g. marsupial mole, marsupial mouse, marsupial wolf, etc.) (Werdelin 1987, Withers *et al.* 2000, Jones 2003, Wroe and Milne 2007), yet never evolved certain advantageous eutherian limb adaptations (e.g. bat wings, whale flippers, deer hooves, etc.). Therefore, understanding how marsupials adapt and specialize their limbs will shed light on how selection acts on development to create morphological novelties and why evolution proceeds along certain paths.

Marsupial and eutherian mammals diverged 124 Mya (Meredith *et al*. 2008) and display very different forms of reproduction. Unlike eutherians, marsupials give birth after a very short gestation period to altricial (under developed) young. Pups are born in an embryonic morphological state (blind, hairless, etc.) but are able to use their robustly developed forelimbs to crawl from the birth canal to attach to the teat (Tyndale-Biscoe 1973, Lillegraven 1975, Shaw and Renfree 2006). This essential crawl requires a strong pectoral girdle and is correlated with a fundamental break between the development of the fore- and hind limb. The forelimb grows rapidly while hind limb growth is delayed (Sears 2009a, Doroba and Sears 2010). As a consequence, strong selection for a robust forelimb has limited adult forelimb variation and constrained marsupial forelimb evolution (Lillegraven 1975, Sears 2004, Cooper and Steppan 2010).

In contrast, there is probably no strong selective pressure on the hind limb during development, as it does not participate in the crawl and undergoes no morphological specialization like the forelimb. Therefore, the hind limb may be exempt from the constraint of the crawl and potentially more morphologically labile than the forelimb. It could also be that the hind limb morphologies are the result of earlier developmental selection pressures for terrestrial locomotion. Schmidt and Fischer (2009) discovered that the forelimb of eutherian mammals is more evolutionarily flexible than the hind limb, as the hind limb has been canalized and constrained from adapting due to requirements for propulsion. This hypothesis of hind limb

constraint makes intuitive sense, as evenly highly specialized mammals such as artiodactyls (antelope, deer etc.) use their forelimbs in a range of functions (i.e. feeding, mating, movement, etc.), while the hind limb is primarily used for propulsion. Schmidt and Fischer (2009) focused primarily on eutherians mammals, however because of the functional requirements associated with the womb-to-teat crawl, study of marsupials hind limbs will test whether the ability for the hind limb to specialize has been constrained as well.

Another consequence of the womb-to-teat crawl on the marsupial body plan has been a reduction in integration between the serially homologous elements (i.e. femur/humerus, radius/tibia, metacarpals/metatarsals) of the limb (Bennett and Goswami 2010, Kelly and Sears 2011). Integration is the phenotypic interdependence of two or more structures, and is estimated by the level of covariation or correlation among structures (Willmore *et al.* 2007). Serially homologous structures (limbs, teeth, vertebrae, etc.) first arose as one structure that was later replicated and repeated (Hall 1995) and therefore ancestrally share common genetic controls and experience high levels of integration. When limb integration is "broken", the limbs become free to evolve separately, potentially allowing morphological radiations to occur (e.g., wings, flippers, etc.; Young and Hallgrímsson 2005). While it has been established that marsupials display a reduced between limb integration in adults (Bennett and Goswami 2010, Kelly and Sears 2011), no study has yet determined integration patterns in developing limbs, for either marsupials or eutherians. It is important to understand the nature of limb integration throughout development because examining integration across contexts (genetic, developmental, functional) can provide information on the evolution of morphologies (Cheverud 1996, Wagner and Altenberg 1996, Klingenberg 2008). Also, even moderate functional requirements (such as the crawl) can influence the developmental plasticity and later adult morphologies (Herring 1993, Enlow and Hans 1996, West-Eberhard 2003).

Therefore, understanding marsupial adult specialization and developmental integration patterns will shed light on the role of the crawl in determining morphological evolution and developmental constraints in both marsupials and mammals in general.

CHAPTER 1: LIMB SPECIALIZATION IN MARSUPIALS[1]

1.1 Introduction

From the bat wing to the whale flipper to the human arm, the form of the limb governs a mammal's range of locomotor, social and feeding behaviors (Polly 2007). As such, the evolution of the mammalian limb complex (i.e., girdles and the limb proper) has played an integral role in the diversification of the group (Oxnard 1963, Wayne 1986, Blob and Biewener 1999, Ji *et al.* 1999, Sears 2004, Polly 2007, Schmidt and Fischer 2009, Tardieu 2010). This is most evident in the dichotomy between the two main groups of extant mammals, marsupials and eutherians.

Despite arising at around the same time, marsupials (kangaroos, opossums, koalas, etc.) and eutherians (humans, dogs, bats, whales, etc.) have experienced very different levels of success, with marsupials today only comprising 6% of modern mammalian species and exhibiting a limited range of girdle and limb morphologies (Lillegraven 1975, Springer 1997, Sears 2004, Cooper and Steppan 2010). In previous research, the relatively reduced morphologic diversity of the marsupial shoulder girdle was linked to constraints imposed by the marsupial newborn's unique functional requirements (Sears 2004), and that of the forelimb proper to a reduced rate of morphological evolution among its distal elements (Cooper and Steppan 2010), in theory also caused by the newborn's functional requirements. These functional requirements come about because marsupials, in contrast to eutherians, give birth after extremely short gestation times to immature neonates that must immediately complete a life-or-death crawl to the teat where they attach and continue to develop (Sharman 1973, Lee and Cockburn 1985, Hughes and Hall 1988, Gemmell *et al.* 2002). This crawl is powered entirely by the forelimb complex, and, consequently, the forelimb complex of the marsupial newborn is advanced in its development (Sears 2009a) and displays a highly modified shoulder girdle to provide the structural support and areas of muscle attachment necessary for the crawl (Cheng 1955, Klima 1987, Sears 2004). The constraint argument is that by having to form the specific crawl morphology at a specific time in ontogeny (i.e., birth) the marsupial forelimb complex is relatively less free to vary its development, and as natural selection acts on existing variation, is

1. This chapter is based on a previous publication: Kelly, E. M. and K. E. Sears. 2011. Limb specialization in marsupial and eutherian mammals: A test of constraints on mammalian limb evolution. In press at *Journal of Mammology*. Permission was received to reprint the material.

divergent functional behaviors (Lillegraven 1975; Sears 2004; Sears 2009a). In contrast to the forelimb, the marsupial hind limb, which plays no role in the crawl, is expected to be freer to evolve and specialize.

This predicted pattern of marsupial limb specialization, with the hind limbs more specialized than the forelimbs, is opposite that recently proposed by Schmidt and Fischer (2009) for mammals. Specifically, they proposed that as the mammalian hind limb is more important for propulsion than the forelimb, the mammalian forelimb should exhibit a higher level of evolutionary flexibility and be more capable of specialization for diverse functions. To test their hypothesis, they examined limb proportions across mammals, and found that, in general, mammalian forelimbs have greater variation in the proportions of their limb elements than hind limbs (Schmidt and Fischer 2009). However, although their ideas are intriguing, their dataset was dominated by eutherians, and they did not explicitly address differences in limb specialization between eutherians and marsupials. Indeed, unlike eutherians, when considering the morphological diversity of both New and Old World marsupials, morphological homoplasy (i.e. convergent evolution) is wide spread, with a relatively few number of body plans representing the majority of marsupials (Lillegraven 1975, Springer 1997, Sears 2004, Cooper and Steppan 2010). Evidence for homoplasy often indicates shared constraint in the presence of similar selective forces (Brooks and McLennan 1991, Brooks 1996, Carrano 2000) and we would expect marsupials to evolve down similar paths (i.e. hind limb specialization) due to the constraints imposed by the crawl.

Therefore, despite the importance of the marsupial constraint to our understanding of mammalian evolutionary history, one of its fundamental corollaries, specifically that marsupials should exhibit less forelimb specialization than eutherians, has never been expressly tested. Furthermore, the relative specialization of the fore- and hind limbs in marsupials and eutherians has not been specifically investigated. Traditionally, specialization has been assumed from measures of morphological diversity (Schmidt and Fischer 2009, Cooper and Steppan 2010), but morphological diversity and limb specialization are not, necessarily, the same. For the purposes of this paper, limb specialization is defined as the morphological evolution of a limb away from the generalized ancestral mammalian limb state (i.e. the morphological adaptation of the bat forelimb for flight). In contrast, morphological diversity (Foote 1994) reflects the overall range

of morphologies realized by a group. To remedy these important gaps in our knowledge, we here quantify fore- and hind limb specialization across mammals. We use these data to test the hypotheses that: (1) marsupials have less specialized forelimbs than eutherians, and (2) marsupials tend to specialize their hind rather than fore- limbs, and eutherians their fore- rather than hind limbs.

1.2 Methods

Data Collection – We measured the maximum length and width (measured mid-way along the shaft along the anterior-posterior axis) of skeletal elements of the girdles (scapula and pelvis), stylopod (humerus and femur), zeugopod (ulna, radius, tibia, fibula) and autopod (metacarpals, metatarsals and phalanges) from 196 therian taxa (82 marsupials, 114 eutherians). However, the autopod was ultimately excluded from analyses, as it was present in less then half of examined specimens (see Appendix 1 for taxa list). We restricted our analyses to limb length and width to maximize homology and thereby comparability among skeletal elements and species. In general, for marsupials one species per genus was examined, and for eutherians one species per family was examined, following the classification of Nowak (1999). We sampled marsupials at a higher intensity because of their lower taxonomic richness relative to eutherians, and to insure that we accurately quantified their full range of limb specialization. More than one species per family or genus were examined if the family or genus contained members of multiple targeted functional groups. In total, we investigated representatives from 21 marsupial families (95% of total marsupial familial diversity) and 97 placental families (84% of total placental familial diversity). The sampled families represent the majority of the morphological and functional variety found within Theria.

Most adult quantitative data was obtained from osteological specimens housed at the Field Museum of Natural History (FMNH) in Chicago, Illinois. However, we also examined specimens housed in the United States at the Smithsonian National Museum of Natural History (Washington, DC) and in the University of California Museum of Paleontology (Berkeley, CA), and in Australia at the South Australian Museum (Adelaide, Australia), the Australian Museum (Sydney, Australia), and the University of New South Wales (Sydney, Australia).

Adult quantitative measurements smaller than 150 mm were sampled with Mitutoyo (Aurora, IL) digimatic calipers, those from 150 mm to 30 cm with Fowler (Des Plaines, IL)

vernier calipers, and those greater than 30 cm with a metric tape measure. Three measurements were taken for every length and width and averaged to minimize measurement error.

Functional Groups – We targeted a range of functional groups that rely on fore- and hind limbs more or less equally in substrate-based locomotion (arboreal quadrupeds [specializing in above branch locomotion] and terrestrial quadrupeds), and hind more than fore- limbs (bipedal saltators). These particular groups were selected because they have representatives from multiple mammalian orders, providing sufficient taxon sampling for statistical analysis. We assigned species to functional groups based on published behavioral descriptions (Nowak 1999), based on their primary mode of locomotion. A result of this was that species that were described as locomoting equally in a terrestrial and arboreal quadrupedal manner were excluded. For each functional group, only one representative from any given genus was included. In total, arboreal quadrupeds were represented by 21 marsupial and 27 eutherian species, terrestrial quadrupeds by 17 marsupials and 33 eutherians, and bipedal saltators by 11 marsupials and 7 eutherians (see Appendix 1 for specific taxa in functional groups).

Data and Analyses – Quantitative measurements were log-transformed prior to analysis to standardize variance (Sokal and Rohlf 1995). A principal component analysis (PCA) including all measurements was performed for marsupials, and another for eutherians. In both analyses, principal component 1 (PC1) was strongly and positively correlated with all variables, suggesting that it is a good proxy for body size. Therefore, to minimize the effect of body size, each length and width measurement (e.g., humerus length) was regressed against the appropriate PC1 (either the marsupial or eutherian), and the resulting residuals used in further analyses unless otherwise noted (Sears 2004, Sears *et al.* 2007). PCA was performed in JMP version 8.0.2 (JMP8).

Correlation matrices were generated for all measured variables for all functional (arboreal quadrupeds, terrestrial quadrupeds, and saltators) groups in JMP 8.0.2. To assess the impact of sampling on the matrices, the original dataset was re-sampled with replacement and the correlation matrices re-estimated 1000 times. The resulting matrices were then compared with the original observed matrix using the mean matrix correlation as an estimate of matrix repeatability, *t* (Marriog and Cheverud 2001). To test whether matrices were significantly similar

to one another, we applied the Mantel's test (9999 replicates). Two matrices were considered significantly similar when the matrix correlation exceeded 95% of randomly generated correlations (Marriog and Cheverud 2001, Goswami and Polly 2010). Matrix correlations were performed using both Pearson's (parametric) and Spearman's (non-parametric) algorithms in "R" using the "Vegan" program (http://vegan.r-forge.r-project.org). These analyses allow us to test our first hypothesis, specifically that marsupials have less specialized forelimbs than eutherians. For data to support this hypothesis, marsupials should display higher correlations between functional groups (e.g., arboreal and terrestrial quadrupeds) than eutherians for forelimb only datasets, consistent with a lower degree of limb specialization in marsupials for specific functional behaviors. These analyses will also allow us to test our second hypothesis, that marsupials tend to specialize their hind rather than fore- limbs, and eutherians their fore- rather than hind limbs. For the data to support this hypothesis, marsupials should display higher correlations between functional groups for their fore- than hind limbs, while eutherians should display higher correlations between functional groups for their hind than fore- limbs.

To provide a visual assessment of limb specialization, we performed two additional PCA's on the log-transformed (but not body size adjusted) data – one for the fore- and one for the hind limb datasets – that included all marsupials and eutherians. We then plotted the resulting PC2 and PC3 for all functional groups (PC1 was excluded because its high correlation with all variables suggests that it reflects size rather than shape) to visualize the relative morphological space occupied by marsupials and eutherians (Foote 1994, Foote 1995, Sears 2004). For our first hypothesis to be supported, marsupial forelimbs should plot closer to the morphospace origin (which is taken to represent the limb morphology of an average, generalized mammal) than those of eutherians, both when all taxa are included and for each functional group. For our second hypothesis to be supported, marsupial hind limbs should plot further from the morphospace origin than marsupial hind limbs, while the opposite should be true for eutherians. However, note that PCA represents a data visualization tool, rather than a statistical test.

We used the absolute distance from centroid for any given taxon as a representation of its degree of limb specialization. To calculate this metric, we calculated the average value for each measurement (e.g., scapula length) for marsupials and eutherians independently. We then subtracted the resulting average value for any given measurement from that of each taxon, and took the result's absolute value. The resulting value represents the distance from the average

value, or centroid, for a given measurement for a given taxa. For each taxon, we then summed the distances from centroid for all measurements. The resulting absolute distance from centroid (hereafter referred to as distance from centroid) represents the morphological divergence of the limbs of the taxon in question from the average mammalian limb, which in this study represents the degree of limb specialization. Distances from centroid were then pooled to allow statistical comparisons of the fore- and hind limbs among marsupials and eutherians as a whole, and among functional groups (i.e., arboreal quadrupeds, terrestrial quadrupeds, and bipedal saltators), using Kruskal-Wallis Test (Sokal and Rohlf 1995). For each marsupial and eutherian comparison (e.g., marsupial vs. eutherian forelimbs), we also performed a combination of bootstrapping and rarefaction to assess the effect that sampling might have on the results (Kowaleski and Novack-Gottshall 2010). To do this, we first subsampled the more speciose group (usually eutherians) at the level of the smaller (usually marsupials). Then, for both the subsampled larger group and the smaller group we resampled the taxa with replacement 1000 times and calculated the mean distance from centroid for every repetition. The resulting mean distance was compared between marsupials and eutherians for each repetition, and a *p*-value was obtained by dividing the number of repetitions in which the original result was upheld by the total number of repetitions (1000). For the data to support our first hypothesis, we should find that marsupial forelimbs have significantly lower mean distances from centroid than those of eutherians for each functional group. For the data to support our second hypothesis, we should find that in marsupials, the forelimb displays a significantly lower distance from centroid than the hind limb, and in eutherians the opposite is the case.

1.3 Results

Marsupial and eutherian limbs display different patterns of correlation – All matrices displayed relatively high repeatability indices (*t*), with values ranging from 0.84 to 0.97, with an average repeatability of 0.93. As results of Spearman's and Pearson's matrix correlations are highly similar, only Spearman's results are discussed here in detail due to the congruence of results. The average correlation among the forelimb matrices of the marsupial functional groups (n = 49, r_s = 0.685) is almost 3 times higher that that of eutherians (n = 67, r_s = 0.233) (Tbl. 1.1 for details). Furthermore, every functional group comparison displays a higher correlation in marsupial than eutherian forelimbs, and all three correlations are significant in marsupials,

whereas only one is for eutherians. The average correlation among marsupial forelimb matrices (n = 49, r_s = 0.685) is also almost twice as high as that of marsupial hind limbs (n = 67, r_s = 0.366). However, the average correlation among the marsupial (n = 49, r_s = 0.366) and eutherian (n = 67, r_s = 0.357) hind limb matrices is very similar. Every marsupial functional group comparison displays a higher correlation in fore- than hind limbs, and while all three of these comparisons are significant in marsupial forelimbs, only two are for the hind limb. In contrast, eutherians display an opposite pattern in which the average correlation among eutherian forelimb matrices (n = 67, r_s = 0.233) is less than (only ~two-thirds) of that of the eutherian hind limbs (n = 67, r_s = 0.357). Moreover, every functional group comparison exhibit a higher correlation in eutherian hind than fore- limbs, and while two of these comparisons are significant in eutherian hind limbs, only one is for the forelimb.

Eutherian limbs occupy more morphospace than marsupial limbs – For every functional group, the morphospaces occupied by eutherian fore- and hind limbs extend further from the origin than those of the comparable limbs of marsupials (Fig. 1.1). This is even the case for functional groups in which the morphospace occupied by marsupials is larger than that of eutherians (e.g., bipedal saltators, Figs. 1.1C and 1.1D). However, there is no obvious general difference in the overall range of morphospace occupation (i.e., overall size of the morphospace cloud) of the fore- and hind limbs of eutherians, or of marsupials.

Eutherian limbs are more specialized than marsupial limbs - For all functional groups, and a comparison of all marsupials and eutherians, the fore- and hind limbs of eutherians show significantly greater distances from centroid than comparable marsupial limbs (forelimb: all taxa, X^2_1 = 160.98, $P < 0.0001$; arboreal quadrupeds, X^2_1 = 56.27, $P < 0.0001$; terrestrial quadrupeds, X^2_1 = 32.77, $P < 0.0001$; bipedal saltators, X^2_1 = 52.35, $P < 0.0001$) (hind limb: all taxa, X^2_1 = 53.06, $P < 0.0001$; arboreal quadrupeds, X^2_1 = 17.51, $P < 0.0001$; terrestrial quadrupeds, X^2_1 = 10.42, $P = 0.001$; bipedal saltators, X^2_1 = 17.05, $P < 0.0001$) (Fig. 1.2A). However, the average difference in distance from centroid between marsupials and eutherians is greater for fore- than hind limbs for every functional group (arboreal quadrupeds: 0.126 for fore- and 0.060 for hind limbs, saltators: 0.210 for fore- and 0.120 for hind limbs, terrestrial quadrupeds: 0.112 for fore-

and 0.081 for hind limbs) and the all marsupial/eutherian comparison (0.142 for fore- and 0.077 for hind limbs).

Eutherian's forelimbs were a greater distance from centroid than their hind limbs for all functional groups, and the analysis of all eutherians (Fig. 1.2B). Of these differences in distance from centroid, two are significant (all eutherians, $X^2_1 = 31.39$, $P < 0.0001$; eutherian arboreal quadrupeds, $X^2_1 = 16.50$, $P < 0.0001$) and two are not (eutherian saltators, $X^2_1 = 2.31$, $P = 0.13$; eutherian terrestrial quadrupeds, $X^2_1 = 2.95$, $P = 0.08$). In contrast, distance from centroid is higher for the marsupial hind than fore- limb for every functional group and within all marsupials. However, these differences are relatively slight when compared to the differences displayed by eutherian limbs (all marsupials, $X^2_1 = 0.96$, $P = 0.33$; marsupial arboreal quadrupeds, $X^2_1 = 0.04$, $P = 0.85$; marsupial terrestrial quadrupeds, $X^2_1 = 1.71$, $P = 0.19$), and are accordingly only significant for one functional group (marsupial saltators, $X^2_1 = 6.83$, $P = 0.01$). All centroid results remained intact after resampling (P values ranged from <0.000 to 0.008 for the forelimb comparisons, and <0.000 to 0.036 for the hind limb).

1.4 Discussion

Although evolutionary constraints have long been proposed as important mediators of morphological diversification (Lillegraven 1975, Alberch 1982; Arthur 2001, Galis *et al.* 2001, Brakefield 2006, Breuker *et al.* 2006, Salazar-Ciudad 2006), documenting their existence has been difficult (Resnik 1995, Beldade and Brakefield 2003, Klingenberg 2003). As a result, relatively few rigorous studies of constraints have been performed (Zelditch *et al.* 1993, Wagner 1995, Polly 1998, Beldade *et al.* 2002, Ciampaglio 2002, Sears 2004, Frankino *et al*. 2005, Cooper and Steppan 2010, Domazet-Loso and Tautz 2010, Kalinka *et al.* 2010). In this study we tested one of the main corollaries of the marsupial constraint hypothesis (Lillegraven 1975, Sears 2004, Cooper and Steppan 2010), namely that marsupial forelimbs should be less free to specialize than marsupial hind limbs and the forelimbs of eutherians. To do this, we used morphometric data from a broad suite of mammals to test the hypotheses that (1) marsupials have less specialized forelimbs than eutherians, and (2) marsupials tend to specialize their hind rather than fore- limbs, and eutherians their fore- rather than hind limbs.

In regard to the first of these hypotheses, we found morphologies of marsupial forelimbs

to be more highly correlated between functional groups than those of eutherian forelimbs, which suggests that marsupial forelimbs are not as specialized for any given functional behavior as eutherian forelimbs. Consistent with this, we found that the morphospace range occupied by marsupial forelimbs is closer to the origin than that occupied by eutherian forelimbs in all comparisons. Furthermore, we also found that eutherian forelimbs had significantly greater morphologic divergence relative to the average mammalian state (as measured by distance from centroid) than marsupial forelimbs, consistent with the forelimb morphology of eutherians being more specialized. Taken together, these results provide strong support for the first of our hypotheses – that marsupials are less likely to specialize their forelimbs than eutherians.

The second hypothesis is easiest to analyze by breaking it into its two components, and addressing separately whether marsupials tend to specialize their hind rather than fore- limbs, and eutherians tend to specialize their fore- than hind limbs. Relevant to the marsupial component, we found that forelimb morphologies of marsupials from different functional groups are twice as correlated as those of their hind limbs. This suggests that morphology varies more between functional groups in marsupial hind than fore- limbs, which is consistent with the hind limb being more specialized for different functional behaviors. In line with this, we found that the morphologic divergence from the average mammalian state was marginally higher for marsupial hind than fore- limbs. In stark contrast to the marsupial pattern, we found that hind limb morphologies of eutherians from different functional groups are more correlated than those of their forelimbs, and that eutherian hind limbs also display a significantly greater morphological distance from the average mammalian condition than eutherian forelimbs. These data are consistent with eutherian forelimbs being more functionally specialized than eutherian hind limbs, and thereby support the second component of the second hypothesis.

When taken together, our results therefore suggest that fundamental differences exist in the mechanistic underpinnings of limb specialization in marsupials and eutherians. This is in line with previous studies that found fundamental differences between marsupial and eutherian limbs in the sequence of limb developmental events (Bininda-Emonds *et al.* 2007, Weisbecker *et al.* 2008, Sears 2009b), developmental growth rates (Sears 2009a), and integration (Goswami *et al.* 2009, Bennett and Goswami 2010, Kelly and Sears 2011). This study's results suggest that when marsupials specialize, they tend to do so in their hind rather than fore- limb, likely because of the constraint on their forelimb from the functional requirements of their newborn's crawl. In

contrast, eutherian specialization tends to preferentially occur in the forelimb, possibly because of the hind limb's functional role in locomotor propulsion, as proposed by Schmidt and Fischer (2009). This suggests that different functional constraints (Alberch 1982, Maynard Smith *et al.* 1985, Schwenk 1995, Richardson and Chipman 2003), one acting very early in life (marsupials) and one much later (eutherians), may have differently channeled the evolution of the limb in today's two main mammalian groups.

However, it is important to note that the average correlation of hind limb morphologies within functional groups is similar in marsupials and eutherians. This suggests that the differences in fore- and hind limb specialization observed in marsupials and eutherians could be largely driven by differences in the capability of the forelimb to specialize in these groups, rather than the hind limb. If this is the case, it would be consistent with a situation in which the evolution of all mammalian hind limbs is constrained because of the hind limb's integral role in locomotor propulsion (Schmidt and Fischer 2009). For marsupials, this would mean that the evolution of both their fore- and hind limbs are functionally constrained, though as a result of different locomotor requirements. Furthermore, if the mammalian hind limb is inherently constrained, then the marsupial forelimb constraint becomes that much more limiting to the evolutionary history of the group. Further testing is needed to tease apart these intriguing scenarios.

In conclusion, our results support the hypothesis that the evolution of the marsupial forelimb complex has been constrained relative to that of eutherians and the marsupial hind limb complex, consistent with studies by previous researchers (Lillegraven 1975, Sears 2004, Cooper and Steppan 2010). Furthermore, our results are consistent with the existence of an additional functional constraint on hind limb evolution across all mammals, and thereby the results of Schmidt and Fischer (2009). Results of this study are also consistent with suggestions of previous authors (Brakefield 2006, Breuker *et al.* 2006) that study of constraints has the potential to bridge developmental systems and evolutionary processes. We advocate future inquiry into this system to sort out the important roles that functional constraints have had on the evolution of the mammalian limb and thereby mammalian evolutionary history.

1.5 Figures and Tables

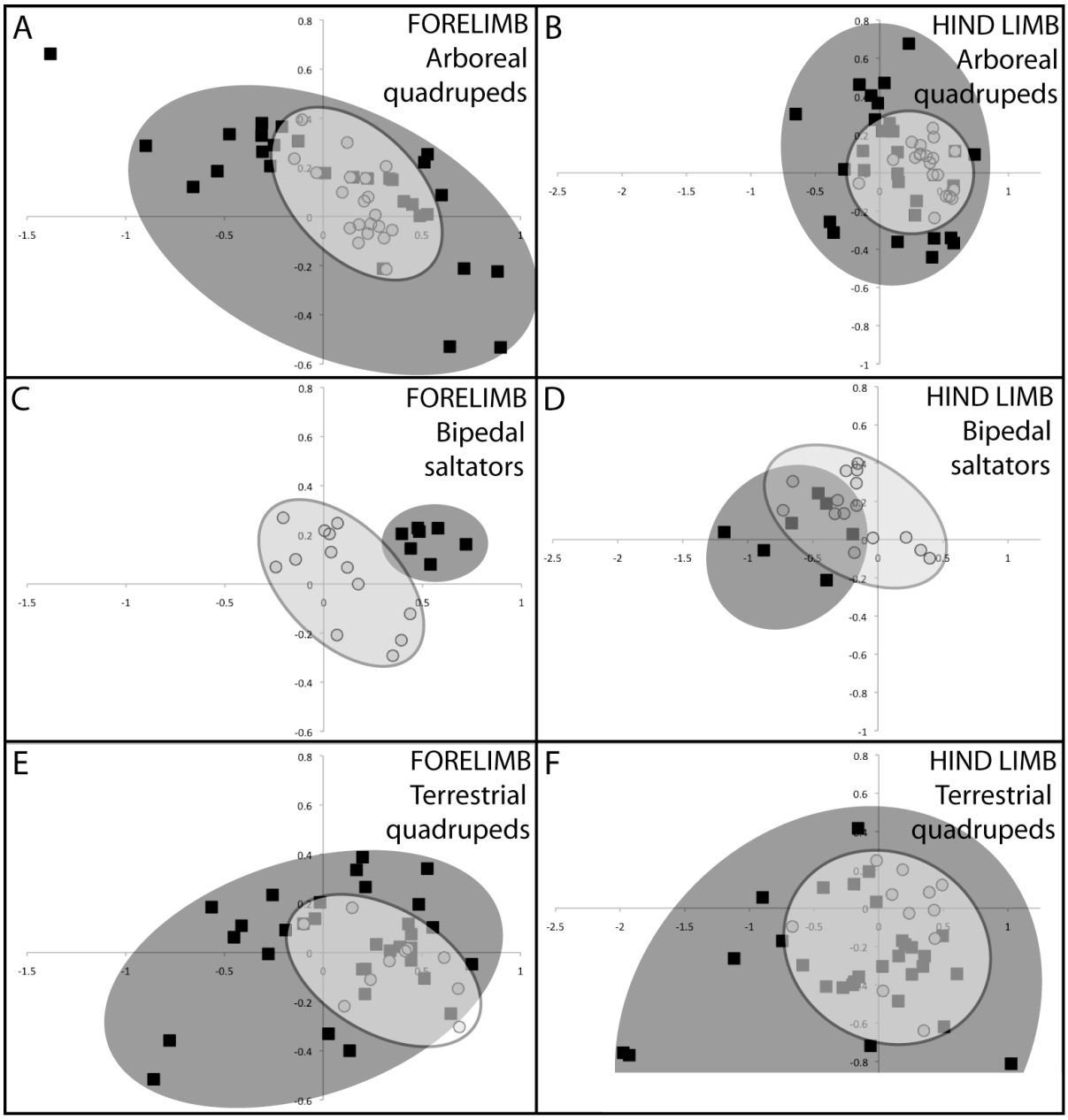

Figure 1.1. Morphospace occupation (plot of PC2 on the x-axis versus PC3 on the y-axis) of marsupial and eutherian fore- (arboreal quadrupeds = A, bipedal saltators = C, and terrestrial quadrupeds = E) and hind limbs (arboreal quadrupeds = B, bipedal saltators = D, and terrestrial quadrupeds = F) by functional group. Light circles and ellipses represent marsupials, and dark squares and ellipses represent eutherians.

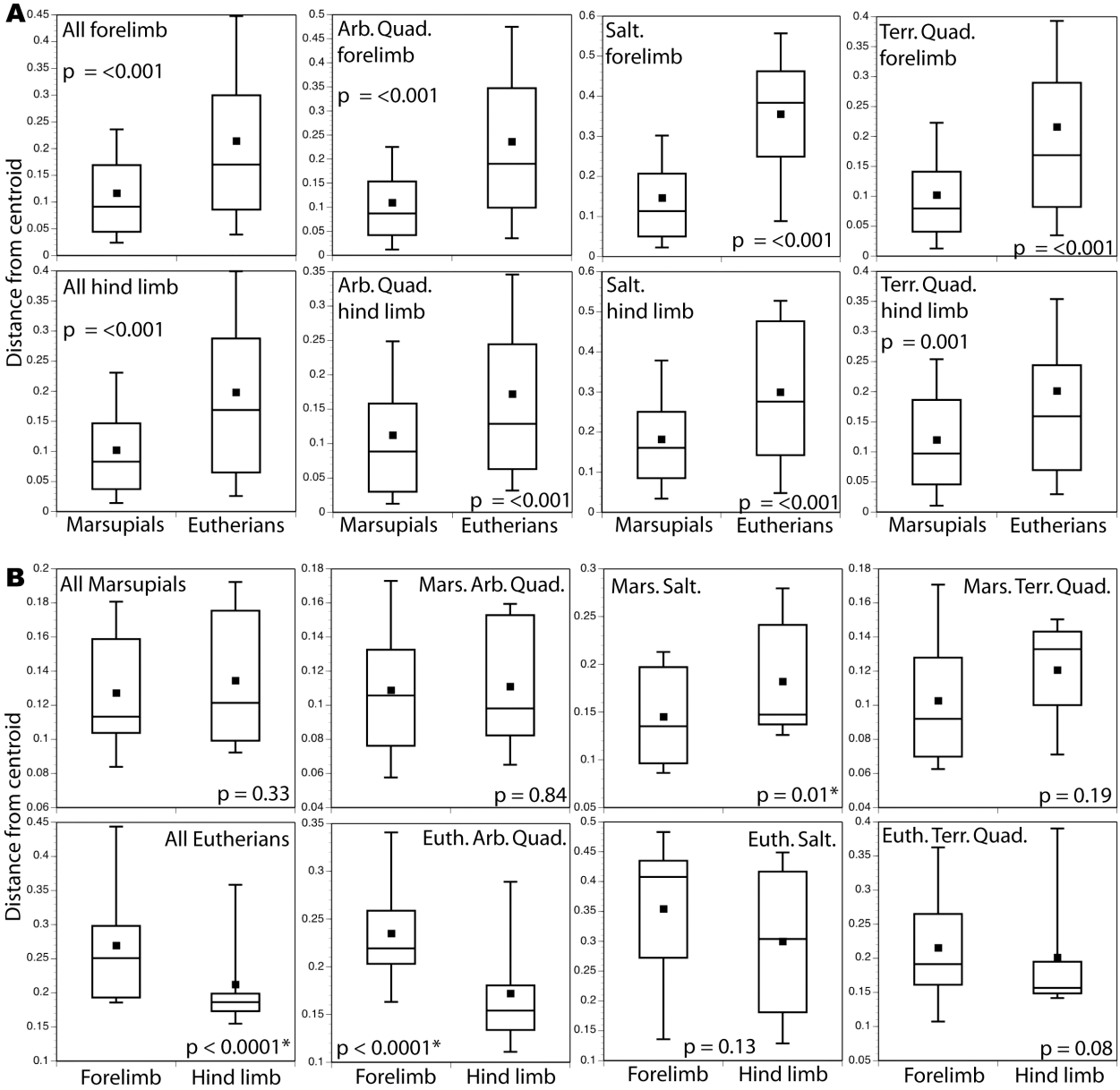

Figure 1.2. Comparisons of the distance from centroid of the fore- and hind limbs within marsupial or eutherian functional groups (A), and of the fore- and hind limbs of marsupials and eutherians by functional group (B). Starred *p*-values are statistically significant at the 0.05 level.

A. Marsupial forelimb				B. Marsupial hind limb			
	Arb. Quads.	Saltators	Terr. Quads.		Arb. Quads.	Saltators	Terr. Quads.
Arb. Quads. (n = 21)	x	0.005*	0.005*	Arb. Quads.	x	0.036*	0.012*
Saltators (n = 11)	0.599	x	<0.001*	Saltators	0.292	x	0.056
Terr. Quads. (n = 17)	0.694	0.762	x	Terr. Quads.	0.505	0.300	x
C. Eutherian forelimb				D. Eutherian hind limb			
	Arb. Quads.	Saltators	Terr. Quads.		Arb. Quads.	Saltators	Terr. Quads.
Arb. Quads. (n = 27)	x	0.102	0.008*	Arb. Quads.	x	0.012*	0.0046*
Saltators (n = 7)	0.313	x	0.543	Saltators	0.356	x	0.279
Terr. Quads. (n = 33)	0.407	-0.022	x	Terr. Quads.	0.598	0.116	x

Table 1.1. Correlations between functional group matrices for marsupial fore- (A) and hind (B) limbs, and eutherians fore- (C) and hind (D) limbs. For A-D, *P* values are located in the upper right of the matrix, Spearman correlation coefficients (r_s) in the lower left. Starred *P* values are statistically significant at the 0.05 level.

CHAPTER 2: DEVELOPMENTAL INTEGRATION IN MARSUPIALS

2.1 Introduction

The vertebrate body plan consists of nested modules that share genetic regulation, covary, and evolve together (Davidson and Erwin 2006, Fujimoto *et al.* 2008, He and Deem 2010). Serially homologous morphological structures (i.e. limbs, teeth, vertebrae etc.) are regulated through pleiotropic control of related genes, leading to genetic integration and coordinated evolution (Cheverud 1996, Villmoare *et al.* 2011). When elements develop and covary together, selection can occur causing a canalization (i.e. buffering of developmental changes, Waddington 1942) of any new and potentially harmful changes to the existing module (Davidson and Erwin 2006, He and Deem 2010). Therefore, studying the evolution of new morphologies that break these canalizing forces is important for understanding not only the functional, genetic, and developmental forces shaping morphology but also the nature of constraint on channeling evolution. The field of integration has looked at the functional and genetic nature of covarying modules but little remains known about developmental integration.

Studies on vertebrate limb integration use adult integration as a surrogate for genetic integration (Leamy 1993, Cheverud 1996, Wagner 1996, Wagner and Altenberg 1996, Klingenberg *et al.* 2003, Young and Hallgrímsson 2005, Klingenberg 2008). Here, integration is defined as the phenotypic interdependence of two or more structures, and is estimated by the level of covariation or correlation among structures (Willmore *et al.* 2007). As the limbs are serially homologous, the three limb segments, the stylopod (femur and humerus), zeugopod (radius / ulna, tibia / fibula) and autopod (carpals / metacarpals / phalanges, tarsal / metatarsal / phalanges) ancestrally share a high degree of integration, exhibited through shared developmental, genetic, and functional controls (Olson and Miller 1958, Hall 1995, Chernoff and Magwene 1999). In the absence of strong selective pressure on one of the limbs, phenotypic similarity and potentially genetic covariation between the limbs will remain strong (Young and Hallgrímsson 2005, Lawler 2008, Villmoare *et al.* 2011). Conversely, when one of the limbs is under strong selective pressure (e.g. on bat forelimbs to become wings), between limb integration is broken and the limbs may diverge phenotypically as well (Young and Hallgrímsson 2005, Sears *et al*. 2006, Weatherbee *et al.* 2006, Cretekos *et al.* 2007, Bennett and Goswami 2010, Kelly and Sears 2011).

The bat, *Carollia brevicaudata*, provides the most well studied model of natural

selection, limb integration, and limb genetics as both adult and developing specimens have been well studied. In a study on adult eutherian limb integration, Young and Hallgrímsson (2005) found the bat (*C. brevicaudata*) to be the only examined species of six with reduced limb integration between limb elements (i.e. humerus and femur) and instead increased integration within limb elements (i.e. humerus and radius). This break in phenotypic covariance between the limbs likely is due to the strong differential selective pressures on the forelimb for powered flight. Numerous studies on the genetic mechanisms of bat limb development provide support for the theory of adult limb integration predicting genetic limb integration, as the underlying genetic controls of limb development are highly divergent in bat fore- and hind limbs (Sears *et al.* 2006, Weatherbee *et al.* 2006, Cretekos *et al.* 2007, Hockman *et al.* 2008, Ray and Capecchi 2008).

While studies on the bat have combined both adult integration and genetic development, most studies on limb integration only study adult skeletal covariance due to the difficulty of obtaining embryos from non-model species. This is unfortunate, as it is important to examine integration across contexts (genetic, developmental, functional) since changes in integration across contexts can provide information on the evolution of morphologies (Cheverud 1996, Wagner and Altenberg 1996, Klingenberg 2008). Though research on patterns of adult limb integration has focused on developmental architecture and genetics as the determinants of adult integration, with support in some species (e.g. the bat) other studies have found a functional basis of adult limb integration patterns. Schmidt and Fischer (2009) found in their study of adult limb integration in 189 mammal species that selection for propulsion on both the fore- and hind limb caused dissociation between serial and functional homologues of the limbs. They discovered the scapula to be functionally analogous to the femur of the hind limb (as opposed to the humerus) and the tarsus and metatarsus to function as a new hind limb element that is analogous to the forearm of the forelimb. Even moderate functional requirements are very important to consider when studying integration as they can influence developmental plasticity of the limbs through processes such as bone remodeling and traits where mechanical load influences the rates and direction of tissue growth (Herring 1993, Enlow and Hans 1996, West-Eberhard 2003).

An even more astonishing finding on limb plasticity and functionality, Losos *et al*. (2000) raised *Anolis sagrei* hatchlings on either broad or narrow surfaces, with the resulting adults significantly differing in hind limb lengths depending on the environment. Their results, along with Schmidt and Fischer (2009) show how environmental pressures influencing different

functional requirements can determine different limb integration as well as demonstrating plasticity in limb morphology in vertebrates at different life stages. Therefore, adult limb integration may not reflect any level of genetic integration and may instead be more affected by post-natal environment. Integration may in fact change throughout the life of an organism, as the limbs experience different selective and functional pressures at different times. Therefore, it is imperative to studied integration throughout ontogeny, to understand the interaction of developmental, selection, and functional requirements in determining adult phenotype.

While most studies on limb integration have focused on eutherians mammals (Young and Hallgrímsson 2005, Lawler 2008, Schmidt and Fischer 2009, Young *et al*. 2010), the less well-studied marsupials are an excellent case study for investigating the functional basis of limb integration throughout the life of an organism, because they experience such different functional requirements of their limbs at different life stages. Eutherians and marsupials differ most greatly in their types of reproduction. Marsupials give birth after a short gestation to altricial young that use their robust forelimbs to make a life-or-death crawl from the birth canal to the teat where they complete their development (Sharman 1973, Tyndale-Biscoe 1973, Lillegraven 1975, Tyndale-Biscoe and Renfree 1987, Hughes and Hall 1988, Renfree 1993, Shaw and Renfree 2006) (Fig. 2.1). This strong selective pressure on the forelimbs to complete a post-natal crawl has led to a reduction in integration between their fore- and hind limbs in both Old and New World species (Bennett and Goswami 2010, Kelly and Sears 2011). This selection on the forelimb for rapid development is also present in the expression patterns of the genes determining early limb growth, with the fore- and hind limb development largely uncoupled (Sears 2009b, Doroba and Sears 2010).

Instead of experiencing a radiation of limb morphologies, the crawl has acted as a constraint on fore- and hind limb evolution in marsupials (Lillengraven 1975, Sears 2004, Cooper and Steppan 2010, Kelly and Sears in press). However, though the majority of marsupials complete the arduous crawl to the teat and display a reduction in phenotypic covariance between the limbs, the patterns of integration within and between the limbs differs between all marsupials species (Bennett and Goswami 2010, Kelly and Sears 2011). This may be due to the variety of different functional requirements their limbs experience, from the forelimb driven crawl to the teat, to the fore- and hind limb grasping of the mother, to the juvenile crawling (both on and off the mother) to a complex adult environment (varying between species)

requiring flexibility in both limbs. As eutherians spend the majority of their development in utero (compared to marsupials) and bypass the crawl from the birth canal, their limbs may experience a less functionally demanding post-natal environment and may not experience extensive changes in their limb integration.

Here, I investigate the role of functional pressure on determining limb integration by examining limb integration in marsupial embryos, both Old World (OW) and New World (NW), with the house mouse as an eutherian outgroup. I included both OW and NW marsupials to determine if limb integration is consistent throughout the group, as may be expected by the constraints of the crawl. This is the first study to determine developing integration as well as the first study to determine if limb integration changes over time. If limb integration is dependent on functional requirements, then the limb integration of different developmental stages will differ. Developmental integration may also differ if the genetic architecture and/or developmental timing differs for the fore- and hind limbs between the limb stages

2.2 Methods

Samples and Data- This study focused on embryos of four species, the NW marsupial the grey short-tailed opossum (Didelphidae - *Monodelphis domestica*, terrestrial quadruped), the OW marsupial the stripe-faced dunnart (Dasyuridae – *Sminthopsis macroura*), the OW marsupial the common brushtail possum (Phalangeridae - *Trichosurus vulpecula*), and the eutherian house mouse (Muridae – *Mus musculus*, terrestrial quadreped). The embryos for *M. domestica* and *M. musculus* were collected from the *M. musculus* and *M. domestica* breeding colonies housed within the Sears Lab (University of Illinois) between 2006 and 2010. The embryos for *T. vulpecula* and *S. macroura* were obtained from the Selwood lab at the University of Melbourne and were collected between 2002 and 2009. The embryos were fixed in 95% EtOH and stored in a 4°C refrigerator before use.

To investigate when developmental integration is laid down and whether it correlates with adult patterns, embryos were chosen that represented the earliest stages of limb growth where each element was represented and later stages of limb growth for comparison. For each species, different but comparable stages were used (Wanek *et al.* 1989), with three stages of *M. musculus* (embryonic day 13.5 n=21, day 15.5 n=22, and day 17.5 n=21), three stages of *M. domestica* (st 33 n=24, PND 1 n=7, and PND 5 n=6), two stages of *T. vulpecula* (PND 2 n=6 and

PND 18 n=6) and one stage of *S. macroura* (PND 6 n=4). Embryos were then eviscerated and skinned before being stained using alcian blue to visualize cartilage growth and alizarin red to visualize bone growth. Protocols for both alcian blue and alizarin red clearing and staining were obtained from the Cretekos lab. Four 3D landmarks were placed on the stylopod (humerus and femur), zeugopod (radius and tibia) and autopod (metacarpal III and metatarsal III) of each specimen using using a reflex microscope (Fig. 2.2).

Analyses- To test whether or not developmental integration is consistent throughout development, correlation matrices were compared among developmental stages within and between species. Correlation matrices were calculated from four landmarks of each limb element, which were then converted to four linear measurements (two along the long axis of the element, two along the short axis). All statistics were calculated using a script written in R, which is available upon request. To allow for comparison between elements, a Procrustes analysis was used to scale all of the elements to unit centroid size. The raw landmark dataset for each species was then resampled 10,000 times using a bootstrap anaylsis and the correlation matrix recalculated. Mantel's test was used to compare correlation matrices for forelimb integration and fore- and hind limb integration between stages of the same species. R^2 values indicate degree of correlation between the stages (with low r^2 values indicating low correlation) while *P* values indicate lack of statistical difference (with *P* values < 0.05 indicating that the two stages do not significantly differ from each other). The correlation coeffecients between pairs of linear measurements (i.e. the length of the humerus and width of the humerus) were then compared between and within limbs and individual elements of the limbs and between species. Mann-Whitney U tests were run to determine whether there were significant differences in covariation within and between the limbs, between stages, and between species. *S. macroura* and *M. domestica* st 33 (with the exception of the femur) were excluded from the between limb and hind limb comparisons as it was impossible to obtain accurate measurements from the hind limbs of the embryos.

2.3 Results

Mantel's test comparison: The results for the majority of the Mantel's comparison of integration at different stages had low r^2 values (indicating low correlation between stages) but were not

statistically different (Figs. 2.3 and 2.4). For *M. domestica*, r^2 values for each stage comparison were low for the forelimb (st 33 to PND 1 r^2=0.368, st 33 to PND 5 r^2=0.403, PND 1 to PND 5 r^2=0.466) (Fig. 2.3) with the post-natal stages more similar to each other than with the pre-natal stage. For the both the fore- and hind limb, r^2 values decreased (PND 1 to PND 5 r^2=0.184) (Fig. 2.4). This may be due to the rapid growth the hind limb exhibits post-birth, as the hind limb completes the majority of its development post-natally. For *M. musculus*, r^2 values for comparisons of the forelimb decreased with age but did not significantly differ (13.5d to 15.5d r^2=0.544, 13.5d to 17.5d r^2=0.446, 15.5d to 17.5d r^2=0.375) (Fig. 2.3). When the hind limb was included, r^2 values decreased even further and showed a slight increase between the later stages (13.5d to 15.5d r^2=0.206, 13.5d to 17.5d r^2=0.207, 15.5d to 17.5d r^2=0.221) (Fig. 2.4). For *T. vulpecula*, the r^2 values for the forelimb only were incredibly low and did significantly differ (PND 2 to PND 17/18 r^2=0.061, *P*=0.272) (Fig. 2.3) and while the r^2 values for the fore- and hind limb integration were also low, they did not significantly differ (PND 2 to PND 17/18 r^2=0.242)(Fig. 2.4).

Correlation matrices comparisons: The matrices for the limb elements for each stage of each species were compared to determine whether or not elements significantly covary (Tbls. 2.1, 2.2, and 2.3). Most of the comparisons for all of the species did not significantly differ in their covariance. However, it is important to note that for *M. domestica* stage 33 the femur significantly differed from the humerus, radius, and metacarpal (*P*=0.002, *P* =0.026, *P* =0.002) (Tbl. 2.1). It is also interesting to note that this decrease in integration between serially homologous elements is not present in the later, post-natal stages of *M. domestica* development (Tbl. 2.1). *M. musculus* 13.5d also exhibited reduced integration between the humerus and femur (*P*=0.041) (Tbl. 2.2). In the later stages of development (15.5d and 17.5d) the humerus and femur did not display reduced integration (Tbl. 2.2).

The phalanges, both fore- and hind limb, of *T. vulpecula* PND 2 significantly differed from all the other limb elements (*P*<0.05) (Tbl. 2.3). Interestingly, PND 2 is very close to birth, when the hind limb is greatly reduced compared to the forelimb. This strong lack of within limb integration was not present in the later stage of *T. vulpecula*.

Mann-Whitney whole limb comparisons: The mean correlation coefficients were determined

using the absolute value of the correlation matrices for within limb integration and between limb integration (Tbl. 2.4, Figs. 2.5 and 2.6). Within limb integration significantly differed for *M. domestica* PND 5 and *M. musculus* 13.5d (P=0.0077, P=0.036) (Tbl 2.4 and Fig. 2.5). Between limb integration significantly decreased for *M. domestica* PND 5 and *M. musculus* 15.5d and *T. vulpecula* PND 2 (P=0.017, P=0.016, P=0.0009) (Tbl. 2.4 and Fig. 2.6).

2.4 Discussion

The vertebrate limb system represents a model for understanding the role of function, genetics, and selection on shaping patterns of integration. Current thought holds that because the limbs are serially homologous structures, the corresponding elements of the fore- and hind limbs should ancestrally share genetic and developmental architecture and covary together (Young and Hallgrímsson 2005). However, there exists no study on an organism's limb integration throughout life even though functional requirements of the limbs can influence developmental plasticity (Herring, 1993, Enlow and Hans 1996, West-Eberhard 2003). As marsupials experience different functional requirements of the limbs throughout their lives, they make an excellent case study for investigating the nature of limb integration from development to adulthood. I predicted that limb integration was dependent on functional requirements and would change throughout the life of an organism. The results of correlation matrices, Mantel's comparisons and Mann-Whitney U tests support this hypothesis as integration between elements, within the fore- and hind limb, and between the limbs changed between the stages (Tbls. 2.1-4 and Figs. 2.3-6). Patterns of limb integration were also highly dependent on species.

Unexpectedly, the patterns of limb integration did not assort between marsupials and eutherians. *M. domestica* was the only marsupial that displayed significantly reduced integration between serially homologous elements (humerus and femur for st 33). As this is the stage immediately before birth, this break in integration between serially homologous elements (the humerus and femur) may be due to the selective pressure of the post-natal crawl made by most marsupial neonates. These integration patterns support those found by Schmidt and Fischer (2009), where the scapula was found to be functionally homologous to the femur, shifting the integration patterns down the limb. During the stages examined (st 33, PND 1 and 5), the possum embryos contain an extensive shoulder girdle used to complete the crawl (Hill and Hill 1955, Sears 2004), which may be functionally integrated with the femur at these stages. After the pups

attach to the teat, the shoulder girdle experiences an extreme reduction until the coracoid is the vestigial element found in adults (Hill and Hill 1955). This decline in the coracoid may be responsible for why marsupials as a whole experience reduced between limb integration as adults, even when the adults are generalized tetrapods (Bennett and Goswami 2010, Kelly and Sears 2011). As the limbs have become dissociated for the crawl, the reduction of the coracoid and re-patterning of the forelimb muscles over time may never allow for a re-integration of the femur and humerus. It would be interesting to determine if the femur covaries with either the coracoid during development, or the scapula in adulthood, as predicted by Schmidt and Fischer (2009).

Surprisingly, the eutherian (*M. musculus*) also displayed significantly reduced integration between serially homologous elements (femur and humerus, 13.5d) and an overall reduction in between limb integration (15.5d). These results do not support the current theory on limb integration, as *M. musculus* is a generalized tetrapod, and therefore theoretically should have strong between limb integration (Young and Hallgrímsson 2005). The early lack of covariance between serially homologous structures may be due to the nature of limb development, where the forelimb starts out slightly ahead of the hind limb (Wanek *et al.* 1989). For 15.5d, the later embryonic reduction in between limb integration may be in preparation for the post-natal environment, where the pups' ungainly sprawling crawl greatly differs from the later upright adult locomotion (*Pers. obs.)*.

The OW marsupial *T. vulpecula* exhibited the largest changes in integration patterns between stages, with the metacarpals and metatarsal significantly differing from the other elements in PND 2. While the results for *T. vulpecula* may be due small sample size bias, the dissociation between the phalanges and other elements may occur as both the manus and pes serve little function this early in the pouch, when the mouth parts have fused to teat (Gemmell *et al*. 2002). This decrease in integration between the manus and pes is not present in the later stage, potentially due to an increase in detaching from the mother to locomote separately, leading to a functional re-integration of the manus and pes with the fore- and hind limbs.*T. vulpecula* also has the longest gestation and gives birth to the largest pups of the marsupials in this study (Gemmell and Hendrikz 1993), which may explain their unusual integration patterns.

This increase in post-natal integration in marsupials (both *M. domestica* and *T. vulpecula*) may also be a product of changes in the hormonal controls of post-birth limb growth. After

birth, the two main regulators of skeletal development are Growth Hormone (GH) and the Insulin-like Growth Factors (IGFs) (Rosenfeld *et al*. 1994, Woods *et al*. 1996, Lupu *et al.* 2001, Rosenfeld and Hwa 2009). However, GH and the IGFs also regulate lipid and carbohydrate metabolism (Lichanska and Waters 2008) and overall tissue growth (Nijhout 2003, Sutter *et al*. 2007, Stanger 2008) and therefore are constantly circulating throughout the body. This continuous and equal presence of GH and IGFs to both the fore- and hind limb regulating growth may be responsible for the increase in covariance between limbs found in the marsupials. It would also explain the level of integration found in the early, post-natal *M. domestica* (PND 1) when the pup is fused to the teat and does not functionally use either the fore- or hind limb.

In light of changes in integration across development, it is interesting to consider the role of limb integration in terms of morphological evolution. The theory proposed by Young and Hallgrímsson (2005) states that when one of the limbs undergoes strong selective pressure, between limb integration will reduce, leading to species radiation. While this is supported in eutherians, both OW and NW marsupials experience strong selective pressure, leading to a reduction in between limb integration that may restrict future adaptive radiations (Bennett and Gowsami 2010, Kelly and Sears 2011). The constraints of a post-natal crawl hold the fore- and hind limbs in check, limiting both the fore- and hind limb's ability to evolve post-crawl (Lillegraven 1975, Sears 2004, Cooper and Steppan 2010). Peculiarly, limb integration does change between stages within marsupials, suggesting that limb integration for marsupials may not play such a strong canalization role as present in eutherians when compared to the crawl. The role of integration as a functional product of the limbs may serve only to modify the existing patterns determined by the limb development required for the crawl. In order to better understand the role of integration in the evolution of limb morphologies, future research should examine the role of developmental integration (both pre- and post-birth) in a wider variaty of species, especially those with novel locomotor skills and when locomotion changes throughout the life of the organism.

Overall, the data clearly show that limb integration changes throughout development, and in marsupials, between limb integration does decrease over time, likely due to the influence of the crawl. Most importantly, decreased between limb integration only allows for specialization in presence of evolutionary force towards specialization and therefore, we should not expect limb radiations due to a reduction of between limb integration, as predicted by Young and

Hallgrímsson (2005). While adult integration patterns may be indicative of genetic patterns of early limb development, the later stages of development and early post-natal growth exhibit fluctuating integration patterns, likely due to the combined effects of differing hormonal control and environment, with new functional limb requirements acting on the new hormonal controls of growth, causing either a decrease or increase of integration in the limbs. It is possible that these later changes in integration actually represent a time of morphological flexibility, where selection can act to prepare the growing limb for their birth environment. There also appears to be a significant difference between marsupial and eutherian developing limb integration, with a continued decline in between limb integration post-birth only in marsupials. This inability to re-integrate the limbs over time in marsupials may be further evidence of the constraints imposed by the crawl, where only minor changes occur within the limbs post-birth. It would be interesting to determine both patterns of integration in highly specialized marsupials (i.e. kangaroos) as well as examine the flexibility of developing eutherians limbs, in a manner similar to Losos *et al.* (2000). Developmental integration should also be investigated in the monotremes, to determine whether post-natal limb flexibility was present in proto-theria and has been lost in marsupials. Indeed, as the results of Losos *et al.* (2000) suggest, post-natal limb flexibility may be basal to vertebrates, highlighting the post-natal environment as a significant time for selection to determine adult form and function.

2.5 Figures and Tables

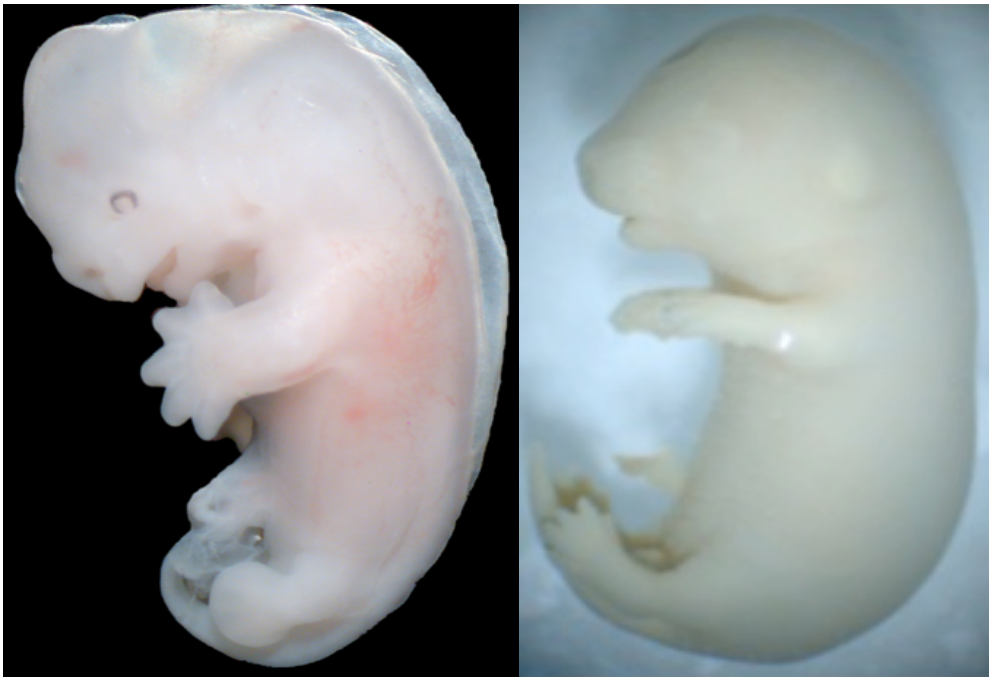

Figure 2.1. Embryos of *Monodelphis domestica* (left) and *Mus musculus* (right) shortly before birth. Note the enlarged forelimb and delayed hind limb of the possum compared to the mouse.

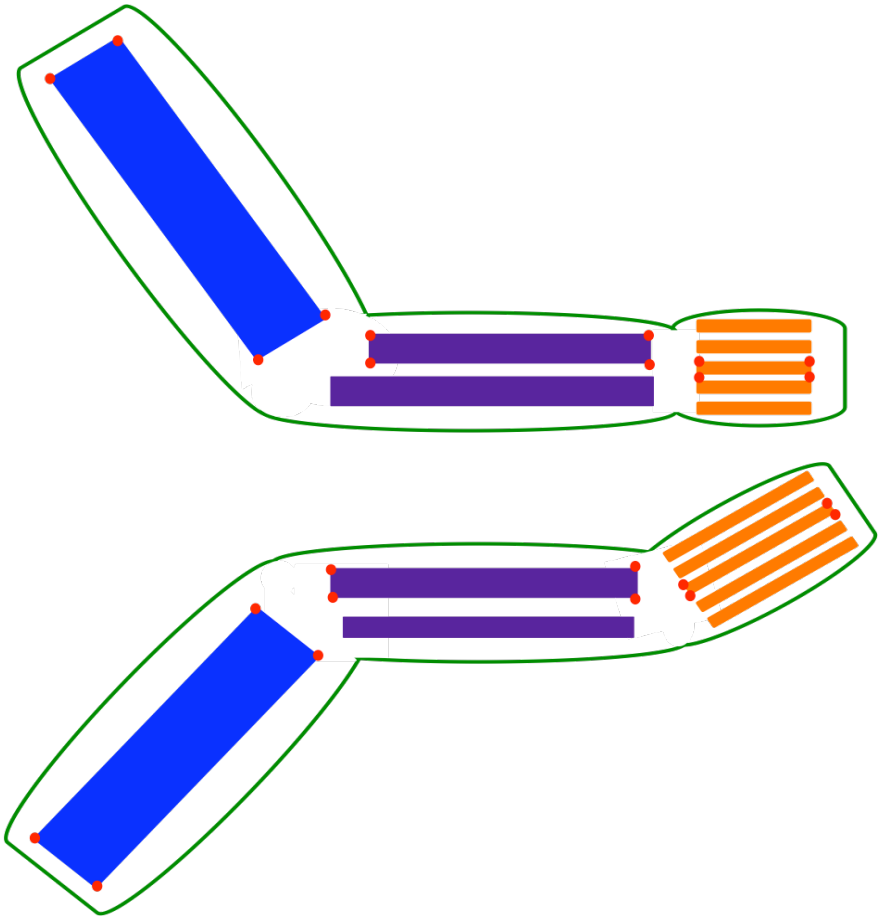

Figure 2.2. Landmarks used to determine developing integration with the forelimb on top. Blue refers to the stylopod (humerus, femur), purple to the zeugopod (radius/ulna, tibia/fibula), and orange to the autopod (metacarpals, metatarsals).

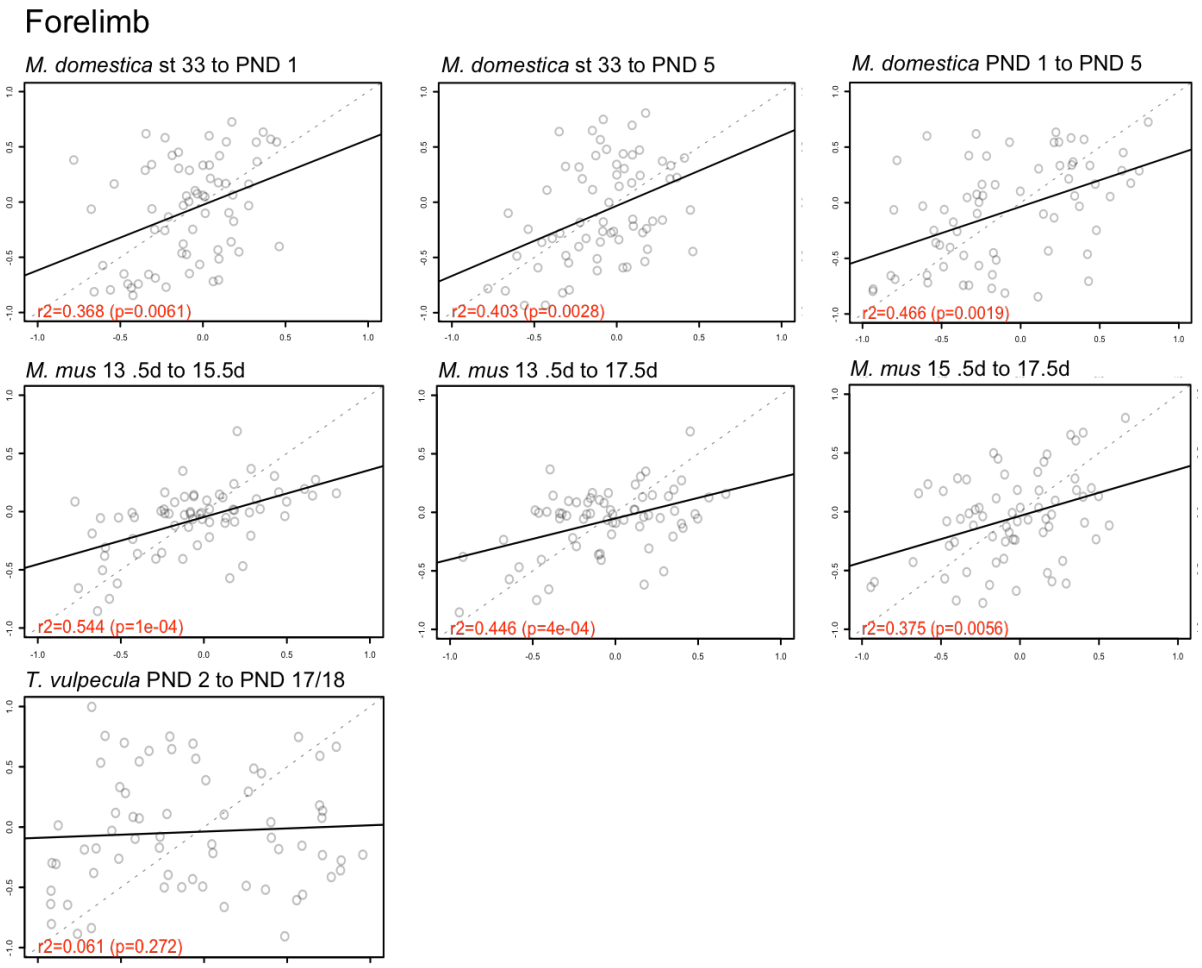

Figure 2.3. Results for the Mantel's test comparison of covariance matrices for forelimb integration between stages. Low r^2 values indicate low correlation between the stages.

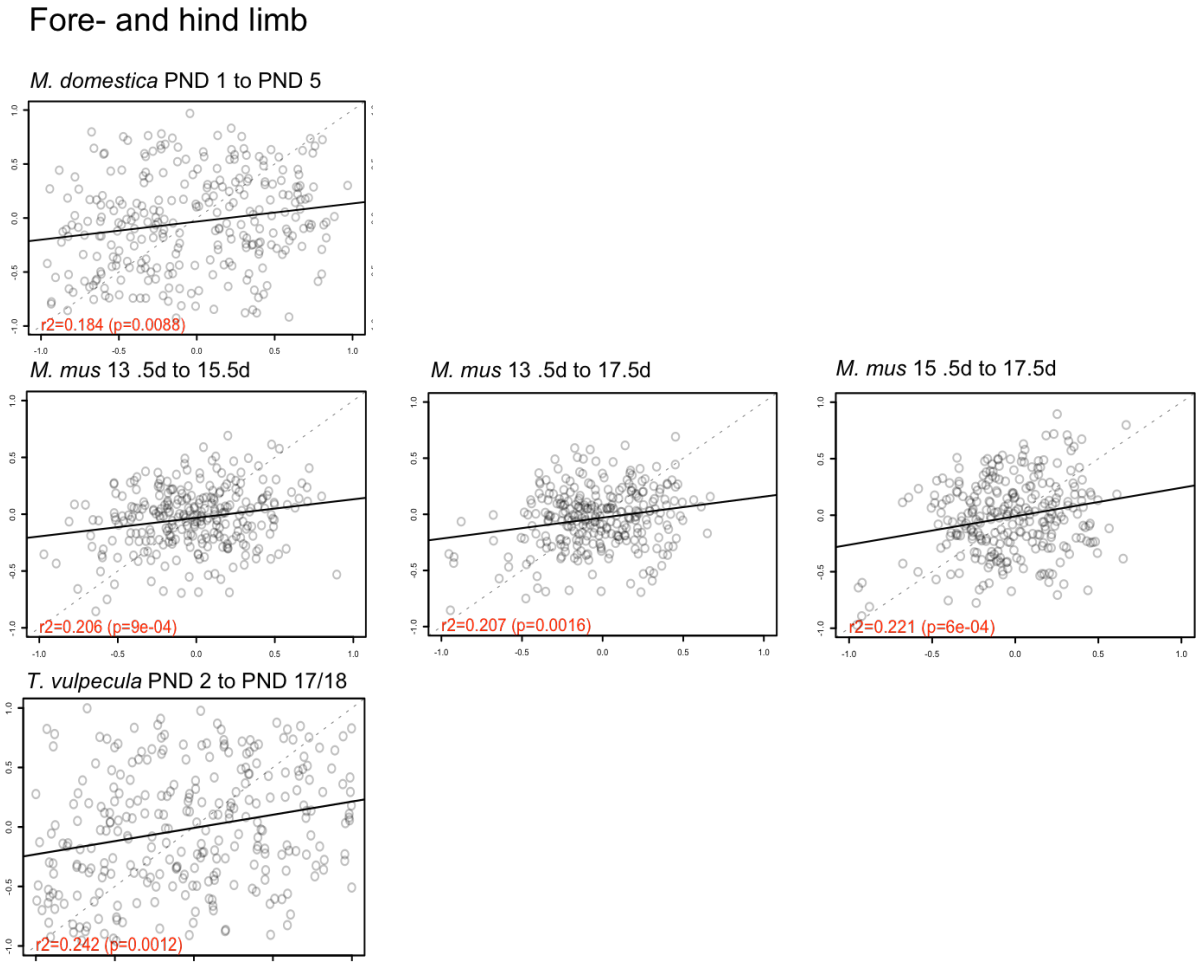

Figure 2.4. Results for the Mantel's test comparison of covariance matrices for fore- and hind limb integration between stages. Low r^2 values indicate low correlation between the stages.

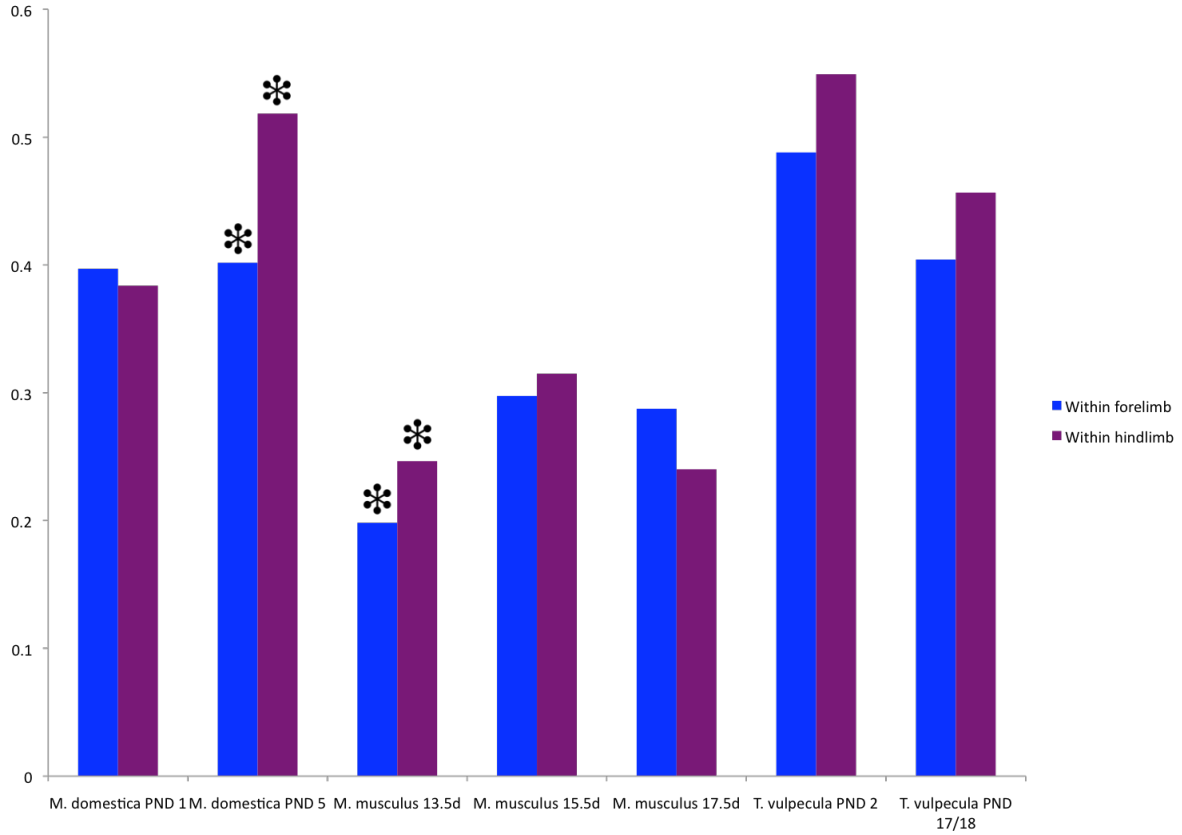

Figure 2.5. Graph of the mean correlation coefficients within limb elements for the fore- and hind limb. Asterisks denote significantly different patterns of integration within the limbs ($P < 0.05$) determined using Mann-Whitney U tests.

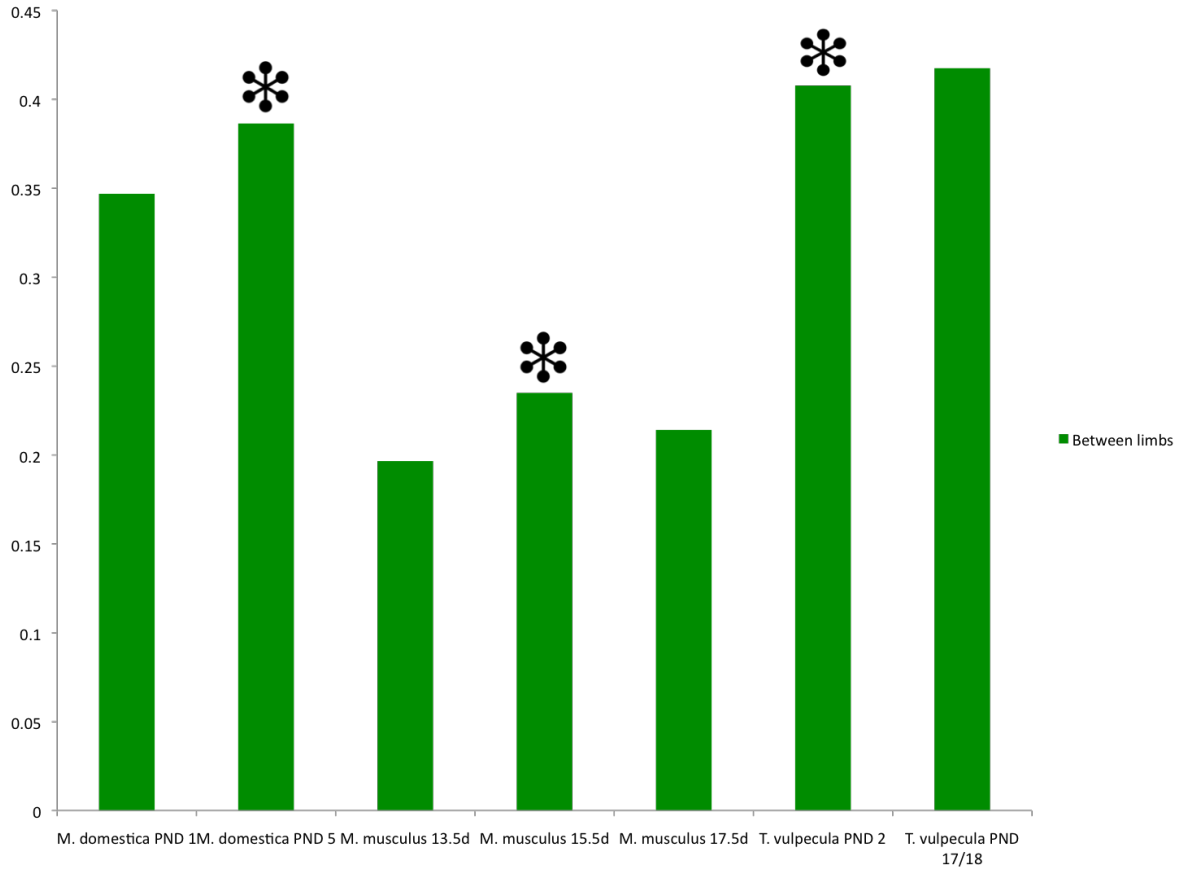

Figure 2.6. Graph of the mean correlation coefficients comparing the correlation of serial homologous elements (humerus to femur) to the correlation of within limb elements (humerus to radius). Asterisks denote significantly different patterns of integration between the serially homologous elements of the limbs ($P < 0.05$) determined using Mann-Whitney U tests.

M. domestica st33		humerus	radius	metacarpal	femur	tibia	metatarsal
	humerus	NA	13.000	13.000	0.000	NA	NA
	radius	0.485	NA	21.000	4.000	NA	NA
	metacarpal	0.485	0.699	NA	0.000	NA	NA
	femur	**0.002**	**0.026**	**0.002**	NA	NA	NA
	tibia	NA	NA	NA	NA	NA	NA
	metatarsal	NA	NA	NA	NA	NA	NA
M. domestica PND 1	humerus	NA	21.000	20.000	21.000	22.000	23.000
	radius	0.699	NA	15.000	17.000	19.000	16.000
	metacarpal	0.818	0.699	NA	20.000	22.000	20.000
	femur	0.699	0.937	0.818	NA	16.000	16.000
	tibia	0.589	0.937	0.589	0.818	NA	18.000
	metatarsal	0.485	0.818	0.818	0.818	1.000	NA
M. domestica PND 5	humerus	NA	26.000	13.000	15.000	17.000	18.000
	radius	0.240	NA	9.000	11.000	11.000	11.000
	metacarpal	0.485	0.180	NA	21.000	19.000	18.000
	femur	0.699	0.310	0.699	NA	19.000	15.000
	tibia	0.937	0.310	0.937	0.937	NA	15.000
	metatarsal	1.000	0.310	1.000	0.699	0.699	NA

Table 2.1. Pairwise comparison of the absolute value of the correlation coefficients between limb elements for three stages of *M. domestica*. The upper triangle is the Mann-Whitney U value and the lower triangle is the p-value. Significantly different comparisons ($P < 0.05$) are marked in red.

M. musculus 13.5d		humerus	radius	metacarpal	femur	tibia	metatarsal
	humerus	NA	28.000	21.000	31.000	15.000	20.000
	radius	0.132	NA	16.000	19.000	9.000	10.000
	metacarpal	0.699	0.818	NA	18.000	15.000	15.000
	femur	**0.041**	0.937	1.000	NA	10.000	9.000
	tibia	0.699	0.180	0.699	0.240	NA	22.000
	metatarsal	0.818	0.240	0.699	0.180	0.589	NA
M. musculus 15.5d	humerus	NA	13.000	11.000	26.000	26.000	18.000
	radius	0.485	NA	11.000	25.000	27.000	20.000
	metacarpal	0.310	0.310	NA	26.000	28.000	24.000
	femur	0.240	0.310	0.240	NA	20.000	11.000
	tibia	0.240	0.180	0.132	0.818	NA	8.000
	metatarsal	1.000	0.818	0.394	0.310	0.132	NA
M. musculus 17.5d	humerus	NA	27.000	17.000	23.000	27.000	21.000
	radius	0.180	NA	8.000	17.000	12.000	16.000
	metacarpal	0.937	0.132	NA	25.000	26.000	23.000
	femur	0.485	0.937	0.310	NA	18.000	15.000
	tibia	0.180	0.394	0.240	1.000	NA	17.000
	metatarsal	0.699	0.818	0.485	0.699	0.937	NA

Table 2.2. Pairwise comparison of the absolute value of the correlation coefficients between limb elements for three stages of *M. musculus*. The upper triangle is the Mann-Whitney U value and the lower triangle is the p-value. Significantly different comparisons ($P < 0.05$) are marked in red.

S. macroura PND 6		humerus	radius	metacarpal	femur	tibia	metatarsal
	humerus	NA	19.000	NA	NA	NA	NA
	radius	0.937	NA	NA	NA	NA	NA
	metacarpal	NA	NA	NA	NA	NA	NA
	femur	NA	NA	NA	NA	NA	NA
	tibia	NA	NA	NA	NA	NA	NA
	metatarsal	NA	NA	NA	NA	NA	NA
T. vulpecula PND 2	humerus	NA	20.000	2.000	18.000	15.000	2.000
	radius	0.818	NA	0.000	13.000	17.000	0.000
	metacarpal	**0.009**	**0.002**	NA	36.000	31.000	15.000
	femur	1.000	0.485	**0.002**	NA	19.000	0.000
	tibia	0.699	0.937	0.041	0.937	NA	3.000
	metatarsal	**0.009**	**0.002**	0.699	**0.002**	**0.015**	NA
T. vulpecula PND 17/18	humerus	NA	18.000	22.000	17.000	13.000	23.000
	radius	1.000	NA	23.000	17.000	12.000	23.000
	metacarpal	0.589	0.485	NA	14.000	9.000	22.000
	femur	0.937	0.937	0.589	NA	14.000	21.000
	tibia	0.485	0.394	0.180	0.589	NA	28.000
	metatarsal	0.485	0.485	0.589	0.699	0.132	NA

Table 2.3. Pairwise comparison of the absolute value of the correlation coefficients between limb elements for one stage of *S. macroura* and two stages of *T. vulpecula*. The upper triangle is the Mann-Whitney U value and the lower triangle is the p-value. Significantly different comparisons ($P < 0.05$) are marked in red.

	Fore- vs. hind within limb integration		Pooled within vs. between limb integration	
	Mann-Whitney_U	*P* value	Mann-Whitney_U	*P* value
M. domestica st 33	**4**	**8.06645E-05**	**854**	**2.87961E-06**
M. domestica PND 1	2233	0.8041054	10478	0.1416524
M. domestica PND 5	**1592**	**0.007705781**	**11081**	**0.01731276**
M. musculus 13.5d	**1716**	**0.03569768**	9622	0.8592049
M. musculus 15.5d	2056	0.5802878	**11098**	**0.0161432**
M. musculus 17.5d	2547	0.09352338	10348	0.2028733
T. vulpecula PND 2	1917	0.2357907	**11696**	**0.000938154**
T. vulpecula PND 17/18	1942	0.2838121	9747	0.7142939

Table 2.4. Mean correlation coefficients taken from the absolute value of the correlation matrices of within and between all elements of each limb per species per stage. Within fore- vs. hind limb compares the within limb integration of the fore- and hind limbs to each other using the Mann-Whitney U test. Pooled within vs. between compares the limb integration of serially homologous elements (humerus to femur) to the limb integration within the limbs (humerus to radius) using the Mann-Whitney U test. Statistically significant results (P <0.05) are in red.

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